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# Deletion of *bglC* triggers a genetic compensation response by awakening the expression of alternative beta-glucosidase

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ABSTRACT

In the plant pathogen *Streptomyces scabies,* the gene *bglC* encodes a GH1 family cellobiose beta-glucosidase that is both required for primary metabolism and for inducing virulence of the bacterium. Deletion of *bglC* (strain Δ*bglC*) surprisingly resulted in the augmentation of the global beta-glucosidase activity of *S. scabies*. This paradoxical phenotype is highly robust as it has been observed in all *bglC* deletion mutants independently generated, thereby highlighting a phenomenon of genetic compensation. Comparative proteomics allowed to identify two glycosyl hydrolases – named BcpE1 and BcpE2 – of which peptide levels were significantly increased in strain Δ*bglC*. Quantitative RT-PCR revealed that the higher abundance of BcpE1 and BcpE2 is triggered at the transcriptional level, the expression of their respective gene being 100 and 15 times upregulated. Enzymatic studies with pure BcpE proteins showed that they both possess beta-glucosidase activity thereby explaining the genotypic-phenotypic discrepancy of the *bglC* deletion mutant. The GH1 family BcpE1 could hydrolyze cellobiose and generate glucose similarly to BglC itself thereby mainly contributing to the survival of strain Δ*bglC* when cellobiose is provided as sole nutrient source. The low affinity of BcpE2 for cellobiose suggests that this GH3 family beta-glucosidase would instead primarily target another and yet unknown glucose-beta-1,4-linked substrate. These results make *S. scabies* a new model system to study genetic compensation. Discovering how, either the *bglC* DNA locus, its mRNA, the BglC protein, or either its enzymatic activity controls *bcpE* genes' expression, will unveil new mechanisms directing transcriptional repression.

## 1. Introduction

In the course of evolution, gene interactions have developed extensively in complexifying genomes, resulting in difficulties to predict the translation of a genotype to a phenotype [1]. Reverse genetics approaches based on targeted gene knockout techniques have encountered unexpected neutral phenotypes of the generated mutants [2], both in prokaryotes and eukaryotes [3–16]. This phenomenon is defined as mutational (or genetic) robustness and occurs potentially at every biological level from macromolecules to complex organisms [17,18]. The term 'Genetic Compensation' (GC) was defined by El-Brolosy and Stainier as "changes in RNA or protein levels that can functionally compensate for the loss of function of another gene" [17]. Three types of mechanisms have been proposed to tentatively explain the absence of – or mild – phenotype subsequent to the inactivation of a gene: the presence of redundant genes (1), alternative biochemical pathways (2), and adaptive mutations (3).

Buffering by gene duplicates (1) is probably the most obvious mechanism expected to account for the compensation of a gene loss. Some pairs of genes – termed 'paralogues' – originate from gene duplication events in which sometimes both copies are retained. This allows them to diverge in function and/or regulation pattern, a process called subfunctionalization, and acts as a major driver for evolution [19–22]. Similarities, but not perfect matches, in regions associated with regulatory motifs were shown to provide the best backup response by a paralogue [23]. Studies on the budding yeast *Saccharomyces cerevisiae* revealed that deletion of a gene that has a paralogue in the genome is more likely to result in a weak or no-effect phenotype than the deletion of single-copy genes [24,25]. This indicates that gene duplication plays a significant role in mutational robustness [14], although its

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contribution to compensation is predicted to be rather small [18,20,24,26]. It is even considered a side effect of other processes responsible for genetic robustness by some authors [7,25,26]. Alternative biochemical pathways (2) or 'backup' pathways [18] are considered more important contributors to genetic robustness. Indeed, the existence of functional diversity and redundancy within biological systems is key to accommodate genetic mutations [18,27,28]. Specialized enzymes involved in distinct cellular processes are expected to take over the role of the missing enzymes even if they have different expression patterns under normal conditions [25]. Another phenomenon which is expected to be even more prevalent in rapidly replicating organisms is genetic adaptations (3). Different types of adaptive mutation are likely to occur depending on the nature of the genetic lesion and sometimes lead to large genomic rearrangements [29]. More subtle and specific adaptive mutations have already been identified in prokaryotic and eukaryotic microorganisms as a consequence of a gene deletion [4,29].

In addition to the three mechanisms presented above, the transcriptional adaptation [17] or TAGC (Transcription Activation Gene Compensation [2]) phenomenon was recently described and explained at the molecular level [15,16]. The sometimes major differences observed between knockout (KO) and knockdown mutants in zebrafish supported research efforts to unveil the molecular basis of such a phenomenon [6]. The disruption of some genes by introducing premature termination codons (PTC) resulted in some cases in genetic compensation. Two distinct research groups reported simultaneously that the compensation observed was due to decaying abnormal mRNAs which triggered the recruitment of chromatin-modification factors to enable the overexpression of compensatory genes, based on their sequence homology to the KO gene [15,16].

In the course of our study of the molecular mechanisms that trigger virulence of the plant pathogen *Streptomyces scabies*, preliminary observations suggested the existence of a genetic compensation phenomenon. Following the deletion of *bglC* encoding the major beta-glucosidase (BG, β-glucosidase) involved in cellobiose consumption, we measured increased total BG activity in the deletion mutant strain Δ*bglC* [30]. In this work, we investigated the robustness of the inconsistency between the genotype and the phenotype of Δ*bglC*, identified the genes and enzymes associated with the increased total BG activity, and revealed that the genetic compensation phenomenon acts by awakening the expression of genes encoding alternative β-glucosidases. The novel case of genotypic-phenotypic discrepancy unveiled in this work is incompatible with the current theories [17], which makes the Δ*bglC* mutant a model system for further investigation on the mechanisms directing genetic compensation.

## 2. Results

### 2.1. Robustness of the compensation phenotype induced by deletion of bglC

Despite the essential function of the cellobiase BglC in directly feeding glycolysis with glucose from cellobiose hydrolysis, the deletion of *bglC* did not prevent growth of *S. scabies* when cellobiose was provided as exclusive nutrient source. Nevertheless, strain Δ*bglC* still displayed a significant growth delay (~36 h) compared to the wild-type strain *S. scabies* 87–22 (Fig. 1A). Experiments performed in liquid culture allowed to quantify that the growth gap was maximal after 48 h with 2- to 3-times lower biomass accumulation by the *bglC* deletion mutant. Δ*bglC* ultimately reached about the same amount of biomass as strain 87–22 but only after seven days of growth (Fig. 1B).

As stated in the introduction, earlier work surprisingly revealed increased total intracellular β-glucosidase activity in strain Δ*bglC* hereby suggesting the existence of a genetic compensation mechanism that could possibly explain the survival of the deletion mutant when cellobiose was provided as the sole nutrient source [30]. To ascertain that the increased global BG activity monitored in strain Δ*bglC* was not due to artifact and/or secondary mutations, other *bglC* deletion mutants were generated by replacing the entire coding region of *scab57721* (*bglC*) by the apramycin resistance cassette, as described previously [30]. Three new *bglC* deletion mutants were isolated and presented a similar feature as the original mutant, Δ*bglC*$^{\#1}$, i.e., about 2- to 3-times increased global intracellular BG activity compared to the activity measured in extracts originating from the wild-type strain *S. scabies* 87–22 (Fig. 2). This result confirmed that the augmented global BG activity as a consequence of deletion of *scab*57721 is a robust phenotype and demonstrates the existence of a genetic compensation mechanism. In the wild-type genetic background of *S. scabies* 87–22, the gene *bglC* (or the product of its transcription, or the BglC enzyme) thus prevents the expression, production and/or activity of one or more alternative β-glucosidases.

### 2.2. Identification of alternative β-glucosidases awakened by bglC deletion

Identification of the enzymes responsible for the observed increased BG activity was performed by comparative targeted proteomics of fractionated BG-positive protein extracts of both wild-type and Δ*bglC* strains. Intracellular soluble lysates of both strains were fractionated by an anion exchange chromatography and fractions obtained were tested against 4-Nitrophenyl β-D-glucopyranoside (pNP-glucose) as substrate to detect those containing β-glucosidases. As shown in Fig. 3A, two distinct peaks were obtained for the extract of the wild-type *S. scabies* 87–22 i.e., the first peak consisting of fractions 21 to 23, and the second peak of fractions 25 to 30.

The BG activities associated with the first peak were absent in the same fractions originating from the extract of the *bglC* deletion mutant suggesting that these BG activities were due to BglC itself. This was confirmed by comparative targeted proteomics which identified signature peptides of BglC only in the fractions from the extracts of the wild-type strain (data not shown). The single β-glucosidase activity peak resulting from the fractionation of the crude intracellular extract of the *bglC* deletion mutant (fractions 25 to 30 in Fig. 3A) corresponds to the second peak observed in fractions of the wild-type extract. However, the measured β-glucosidase activity is about 10 times higher in fractions originating from the *bglC* deletion mutant (see fraction 27 in Fig. 3A) suggesting that the latter should contain enzyme(s) responsible for the observed increased activity on pNP-glucose in the extracts of strain Δ*bglC*.

Fractions of peak 2 were analyzed by comparative targeted proteomics and signature peptides of two candidate glycosyl hydrolases (GH) were found to be significantly overrepresented in extracts of the *bglC* deletion mutant compared to extracts of the wild-type *S. scabies* 87–22 (Fig. 3B). These two proteins were SCAB_2391 (3 signature peptides) and SCAB_64101 (4 signature peptides) (Fig. 3B). SCAB_2391 and SCAB_64101 were named BcpE1 and BcpE2, respectively, for BglC compensating enzyme (1 and 2).

The protein sequence of *scab2391* (BcpE1) is a 496-amino acids and 54 kDa enzyme that, like BglC, belongs to the GH1 family. Both proteins share 60.7% and 72.2% amino acid identity and similarity, respectively. The *scab2391* open-reading frame (ORF) is part of an operon whose organization is similar to the *cebR-cebEFG-bglC* operon, making *bcpE1* a paralogue of *bglC*. The protein sequence of *scab64101* (BcpE2) is an 821-amino acids and 86.7 kDa enzyme belonging to the GH3 family that includes enzymes with diverse substrate specificities including β-D-glucosides, β-D-xylosides and *N*-acetyl-β-D-hexosaminides among others and involved in many metabolic pathways [31].

### 2.3. Enzymatic properties of BcpE1 and BcpE2

Enzymatic activity assays with pure histidine-tagged BcpE1 and BcpE2 proteins (Fig. 4A) confirmed the ability of both GHs to hydrolyze pNP-glucose (Fig. 4B). This result confirmed that both enzymes identified by the targeted proteomic approach were indeed the most likely

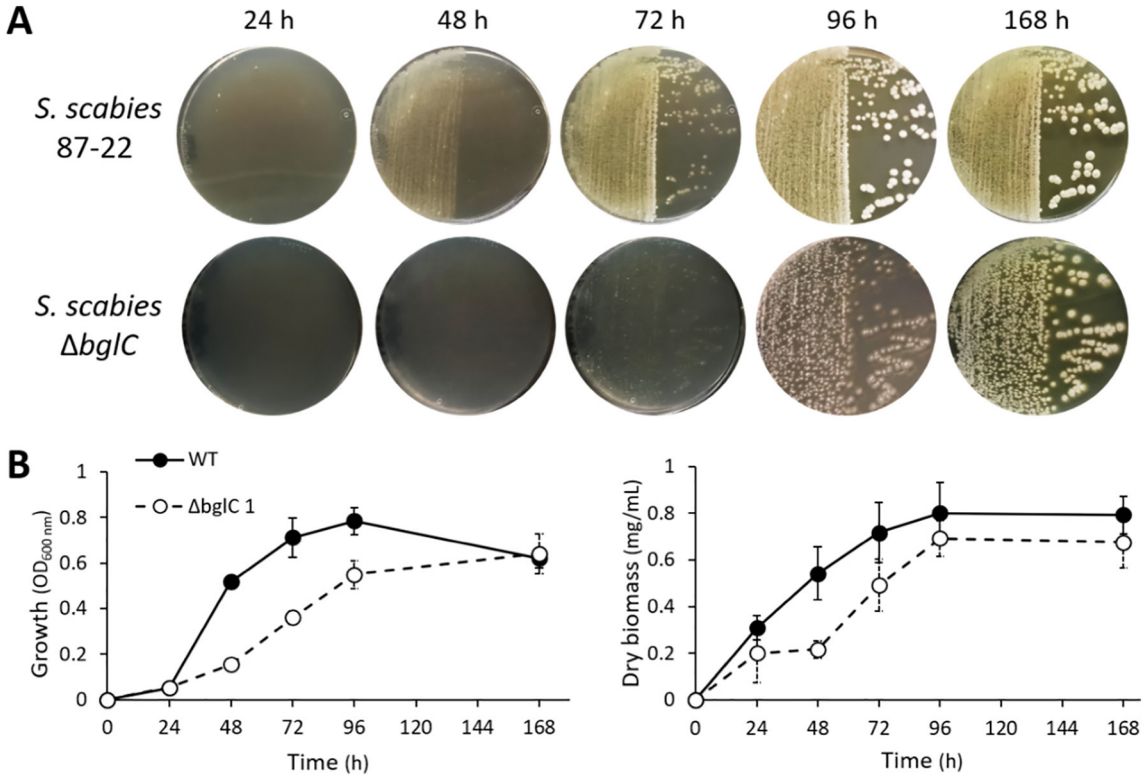


**Fig. 1.** Growth delay and growth deficiency caused by the deletion of *bglC* in *Streptomyces scabies*. A. Impaired growth/growth delay of the Δ*bglC* mutant observed on solid TDM + cellobiose 0.7% medium. B. Quantification of the growth gap between the wild-type strain *S. scabies* 87–22 and its Δ*bglC* mutant in liquid TDM + cellobiose 0.7% assessed by optical density ($OD_{600\ nm}$) (left panel), or dry biomass (right panel).

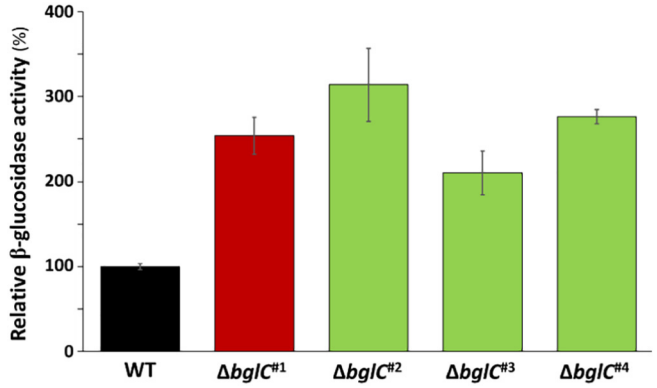


**Fig. 2.** Increased intracellular β-glucosidase activity of *S. scabies* resulting from *bglC* deletion. Relative values of the different *bglC* deletion mutants (Δ*bglC* strains) were compared to the β-glucosidase activity of intracellular extracts of the wild-type strain *S. scabies* 87–22 (WT) which was fixed to 100%. BG activity was measured using pNP-glucose as substrate. Standard deviations were calculated on three technical replicates. The BG activity of the three newly generated *bglC* deletion mutants is highlighted in green.

candidates for the high β-glucosidase activity observed in the extracts of the Δ*bglC* mutants. However, pNP-glucose was hydrolyzed about 10 times more efficiently by BcpE2-$His_6$ than by BcpE1-$His_6$ (Fig. 4B), suggesting that the former is responsible for most of the measured BG activity of peak 2 in Fig. 3A.

In addition, BcpE1-$His_6$ and BcpE2-$His_6$ were tested for GH activity against cellobiose as substrate by Thin Layer Chromatography (TLC) assays (Fig. 4C), confirming the ability of both BcpE enzymes to degrade cellobiose into glucose. However, in this case, BcpE1-$His_6$ appeared to be much more efficient compared to BcpE2-$His_6$ at releasing glucose from cellobiose. BcpE1-$His_6$, like BglC [30], was also able to release glucose from cellotriose (three β-1,4-linked glucose residues), and lactose (glucose β-1,4-linked to galactose) though with much less efficiency (Fig. 4C). Characterization of kinetic parameters of BcpE1-$His_6$ and BcpE2-$His_6$ was performed by determining the initial rate of cellobiose hydrolysis (glucose release) at various concentrations of cellobiose. BcpE1-$His_6$ showed a maximum reaction rate (Vmax) of $0.35 \pm 0.01\ mM.min^{-1}$ (corresponding to $9.12\ \mu mol.min^{-1}.mg^{-1}$). The $K_m$ and $k_{cat}$ values were $1.14 \pm 0.19$ mM and $501 \pm 11.5\ min^{-1}$, respectively (Fig. 5). These values are in the same order of magnitude as those measured for BglC [30] suggesting that BcpE1 is indeed an alternative cellobiase. In contrast, the kinetic parameters could not be determined for BcpE2-$His_6$ as the maximal reaction rate could not be reached (Fig. 5A) suggesting that cellobiose is most-likely not a natural substrate of this GH3 family β-glucosidase.

### *2.4. Compensation is mediated via transcriptional activation of bcpE1 and bcpE2*

A targeted proteomic approach revealed higher accumulation of peptides of BcpE1 and BcpE2 in extracts of the *bglC* deletion mutant (Fig. 3). To determine whether the overproduction of BcpE1 and BcpE2 originated from the transcriptional activation of the ORF *scab2391* and *scab64101*, respectively, we compared the expression levels of both genes by Quantitative Reverse Transcriptase PCR (qRT-PCR). Total RNA was collected from *S. scabies* 87–22 and strain Δ$bglC^{\#1}$ incubated for 1 h in MM with cellobiose 500 μM supplied as the only carbon source. As shown in Fig. 6, the expression of both *bcpE* genes was drastically increased in Δ*bglC* compared to the wild-type strain with *bcpE1* and *bcpE2* displaying about 106-fold and 15-fold increased expression, respectively. As expected, no transcription signal could be detected for *bglC* in mRNA collected from Δ$bglC^{\#1}$ mutant. This result demonstrates that the genetic compensation resulting from the deletion of *bglC* involves a mechanism that activates the transcription of

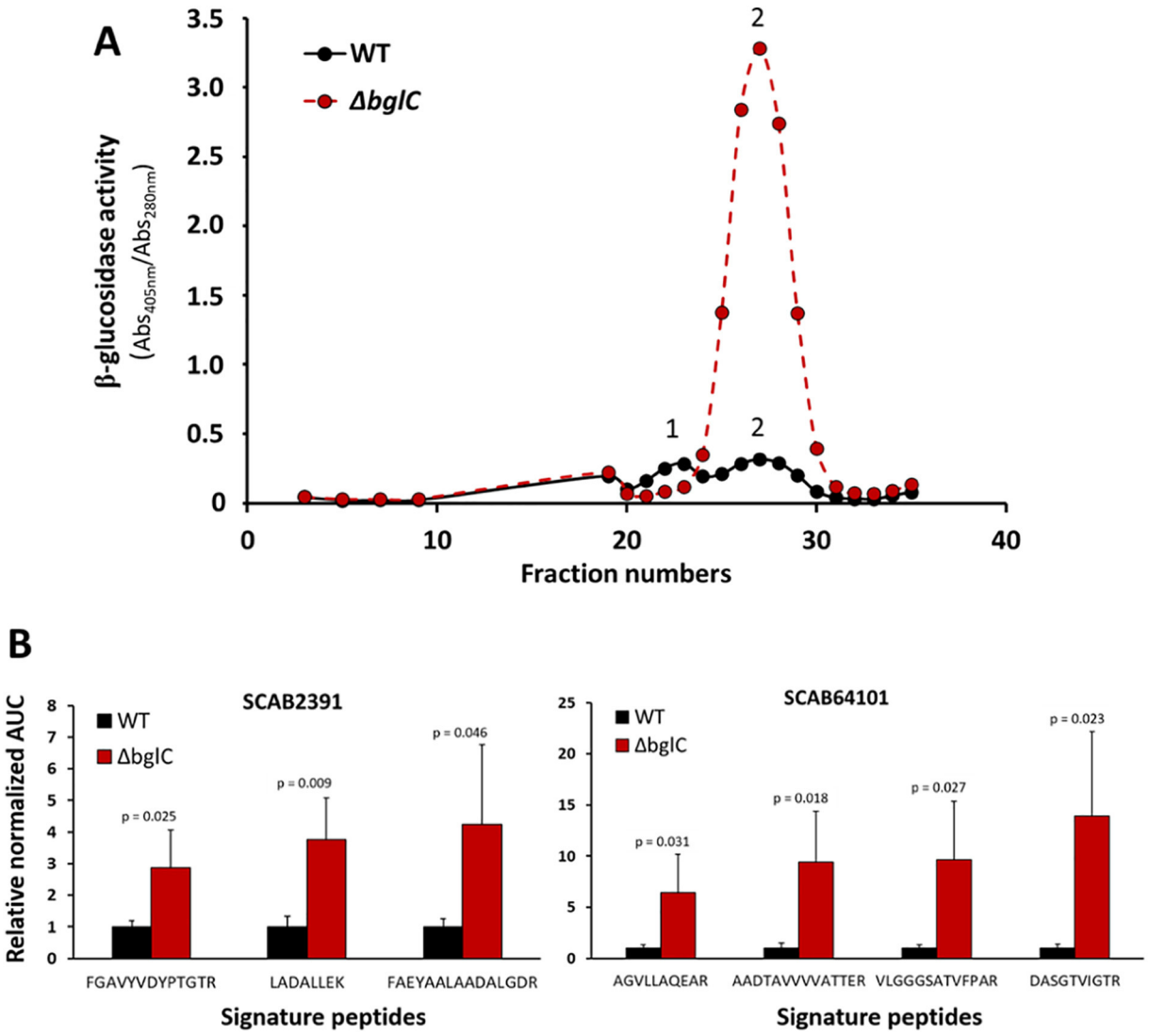


**Fig. 3.** Identification of alternative β-glucosidases overproduced following *bglC* deletion. A. β-glucosidase activities of fractions resulting from anion exchange chromatography of crude extracts of strains *S. scabies* 87–22 (WT) and Δ*bglC*. For each fraction, the relative β-glucosidase activity is expressed as the ratio of the absorbance at 405 nm ($Abs_{405nm}$) resulting from pNP release, according to the total protein content ($Abs_{280nm}$). Fractions 21 to 23 form the first peak (1), and fractions 25 to 30 from the second peak (2). B. Targeted proteomics on fractions 25 to 30. Relative normalized abundance of BcpE1 and BcpE2 proteotypic peptides in response to the deletion of *bglC* (Δ*bglC*) determined by Liquid Chromatography – Multiple Reaction Monitoring (LC-MRM) mass spectrometry (MS) on tryptic digests of protein extracts. AUC, Area Under the Curve. Signature peptides shown were significantly more abundant ($p < .05$) in Δ*bglC* compared to the WT strain.

alternative β-glucosidase encoding genes.

## 3. Discussion

The inactivation by gene deletion of *bglC* encoding a cellobiose β-glucosidase that is key for the onset of the pathogenic behavior of *S. scabies* allowed us to uncover a novel case of genetic compensation. Importantly, we confirmed the robustness of the previously reported paradoxical phenotype [30] by analyzing multiple *bglC* deletion mutants which all displayed increased β-glucosidase activity (Fig. 2). Our results unambiguously prove that a mechanism is in place to safeguard the loss of function of BglC by activating the expression of genes (*bcpE1* and *bcpE2*) whose products, each with different efficiency, can use cellobiose. That the genetics of *S. scabies* has evolved to minimize the impact of the accidental loss of BglC is somehow straightforward to understand knowing the capital developmental importance of cellobiose and cellotriose for this organism ([30,32–38]). BcpE1 is a much better "compensating" enzyme compared to BcpE2 regarding its ability to release glucose from cellobiose or cellotriose. The kinetic parameters of BglC and BcpE1 display values with similar order of magnitude, yet, although BglC has a slightly better affinity for cellobiose, BcpE1 has higher turnover and Vmax values. Instead, cellobiose hydrolysis by BcpE2 is marginal suggesting that its contribution to the survival of strain Δ*bglC* when cultivated with cellobiose as the sole nutrient would be minor compared to BcpE1. The different cellobiose hydrolyzing capabilities of each compensating enzyme is also in line with the fact that BglC and BcpE1 both belong to the GH1-family of glycosyl hydrolases while BcpE2 belongs to the GH3 family. In addition, enzymes belonging to GH1 family would be more promiscuous regarding substrate specificity compared to GH3 and therefore BcpE1 would naturally be a better candidate enzyme for compensating the loss of a function. However, the higher activity of BcpE1 compared to BcpE2 is most likely because *bcpE1* is a paralogue of *bglC* with both genes included in operons presenting identical synteny and both proteins sharing 60.7% and 72.2% amino acid identity and similarity, respectively. Finally, RNA-seq data from a transcriptomic study also support the idea that *bcpE1* (but not *bcpE2*) would be at least partly dedicated to support the product of *bglC* in cellobiose consumption. Indeed, upon

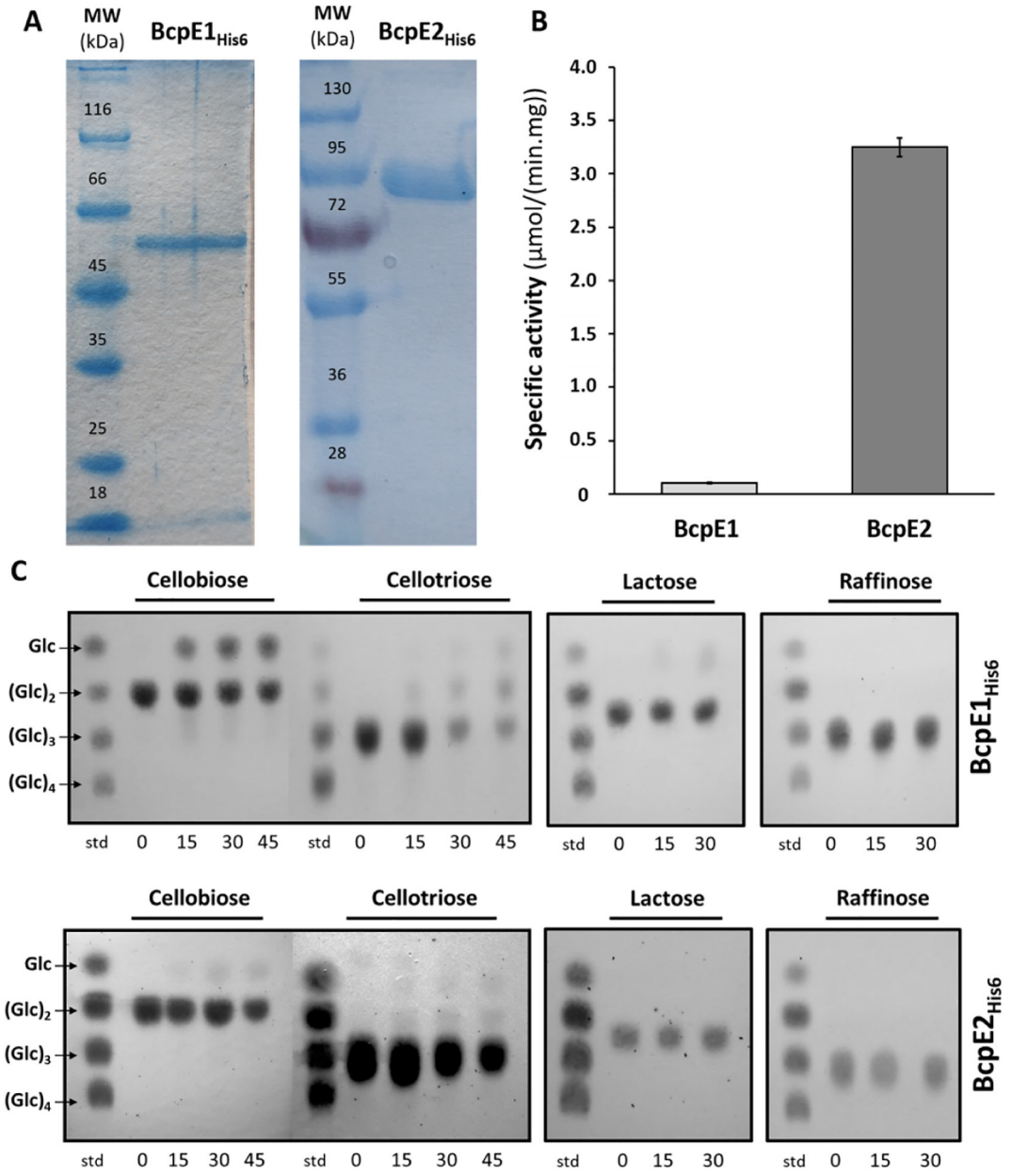


**Fig. 4.** BcpE1 and BcpE2 are active β-glucosidases. A. SDS-PAGE with purified BcpE1-$His_6$ (left) and BcpE2-$His_6$ (right) obtained by heterologous production and subsequent purification by nickel affinity chromatography. B. Specific activity (µmol.$min^{-1}$.$mg^{-1}$) of BcpE1-$His_6$ and BcpE2-$His_6$ against pNP-glucose (1 mM) at 25 °C. C. TLC analysis of cello-oligosaccharides and other oligosaccharides hydrolysis by BcpE1 (top panels) and BcpE2 (bottom panels). 9.37 mM of oligosaccharides were incubated with pure enzyme (0.4 µM) at 30 °C for 0, 15, 30 and 45 min. Std.: Standard cello-oligosaccharides, Glc: glucose, $(Glc)_2$: cellobiose, $(Glc)_3$: cellotriose, $(Glc)_4$: cellotetraose.

cellobiose supply, *S. scabies* 87–22 overexpresses both *bglC* (~220-fold) and *bcpE1* (~16-fold) while *bcpE2* tends to be down-regulated. Also, analysis of the relative basal expression shows that *bcpE1* has ~7-times less mRNA and limited importance in cellobiose response compared to *bglC* in the wild-type genetic background, suggesting that the latter encodes the primary enzyme dedicated to cellobiose hydrolysis (Deflandre et al., manuscript in preparation).

Regarding the role of BcpE2, we hypothesize that it would be primarily involved in targeting another type of glucose-β-1,4-linked substrate than cellobiose. Yet, the high expression level and the high BG activity of BcpE2 suggest that this enzyme is still a major player in strain Δ*bglC*. The *bglC* deletion mutant also displayed a series of unexpected phenotypes that do not fit with the current model of pathways involved in triggering the virulence of *S. scabies* [30]. Among the inexplicable phenotypes, strain Δ*bglC* constitutively produces – whichever culture medium used – the thaxtomin herbicides, the main virulence determinants of *S. scabies*. How the loss of *bglC*/BglC could affect thaxtomin biosynthesis also in culture conditions where the substrates of this enzyme – cellobiose and cellotriose – are absent, is a total mystery. This could possibly be a consequence of the higher expression and therefore higher activity of BcpE2 (and BcpE1) on their natural substrate and not cellobiose. As preliminary evidence of a role of BcpE enzymes beyond cellobiose consumption, results from a proteomics study comparing *S. scabies* wild-type 87–22 and Δ*bglC* grown on ISP4 agar medium (thus not containing cellobiose) also revealed an overproduction of 2.4- and 4.5-fold of BcpE1 and BcpE2, respectively (B. Devreese, S. Planckaert personal communication). This result demonstrates that the transcriptional activation of the *bcpE* genes is not restricted to growth conditions requiring cellobiose catabolism for survival. The morphology of the *bglC* deletion mutant is also atypical

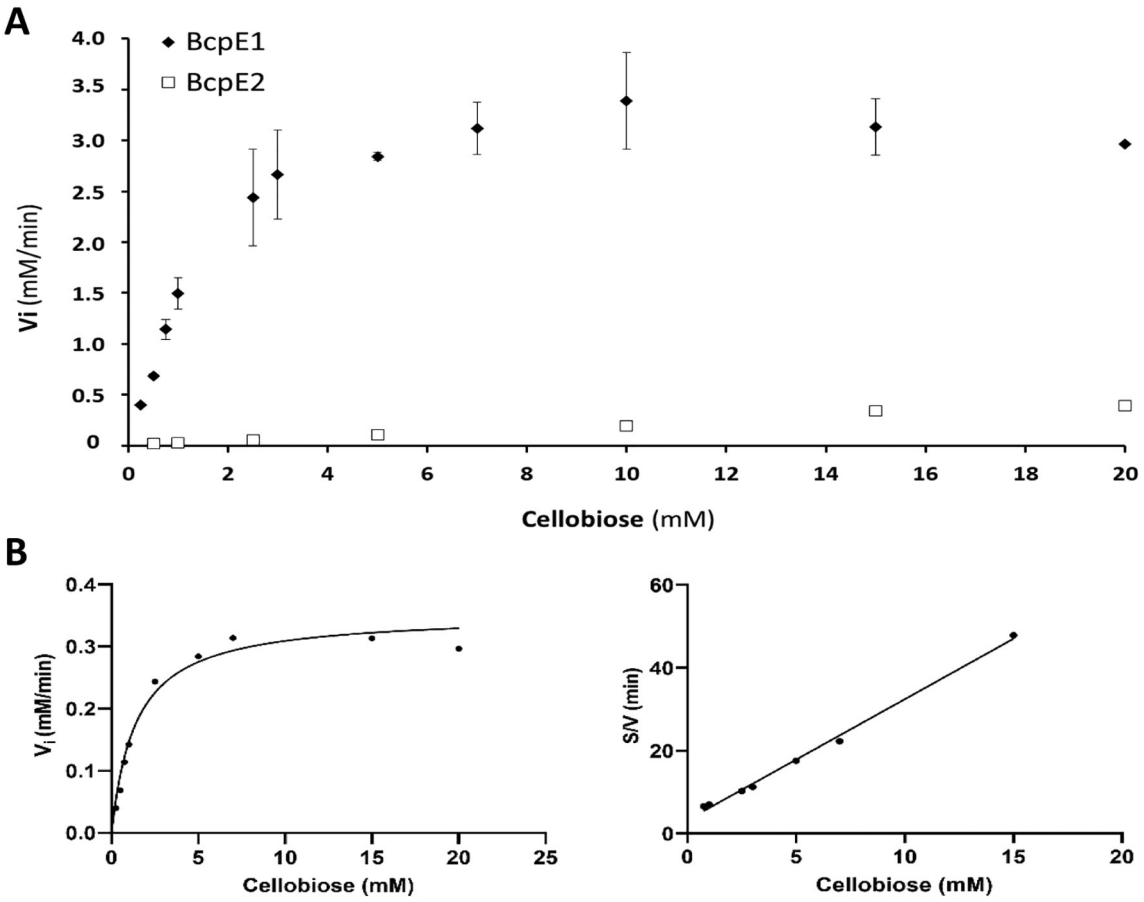


**Fig. 5.** Kinetics of the cellobiose degrading properties of BcpE1 and BcpE2.
A. Kinetic properties of BcpE1-His$_6$ and BcpE2-His$_6$ against cellobiose. Curve of initial velocity (Vi) of BcpE1-His$_6$ and BcpE2-His$_6$ at different concentrations of cellobiose. Rates of cellobiose degradation were obtained by measuring released glucose (see Materials and methods section for details). B. Fitting of the data from (A) for BcpE1-His$_6$ to the Henri-Michaelis-Menten equation using the GraphPad Prism 8 software (left panel) and Hanes linearization (right panel). Equation: y = 2.849 x + 3.264.

with highly fragmented mycelium, a phenotype that resembles those of mutants affected in the synthesis of the cellulase-degradable extracellular polymer (EPS) [39–41]. BcpE2 could potentially participate in EPS degradation.

Genetic robustness examples have already been described but most haven't been investigated in depth. Although recent work highlighted the molecular basis of a particular genetic compensation mechanism involving Pre-mature Termination Codons (PTC)-mediated mutagenesis in model eukaryotes [15,16], many other cases based on other mutagenesis methods remain unexplained [17]. Indeed, the generation of

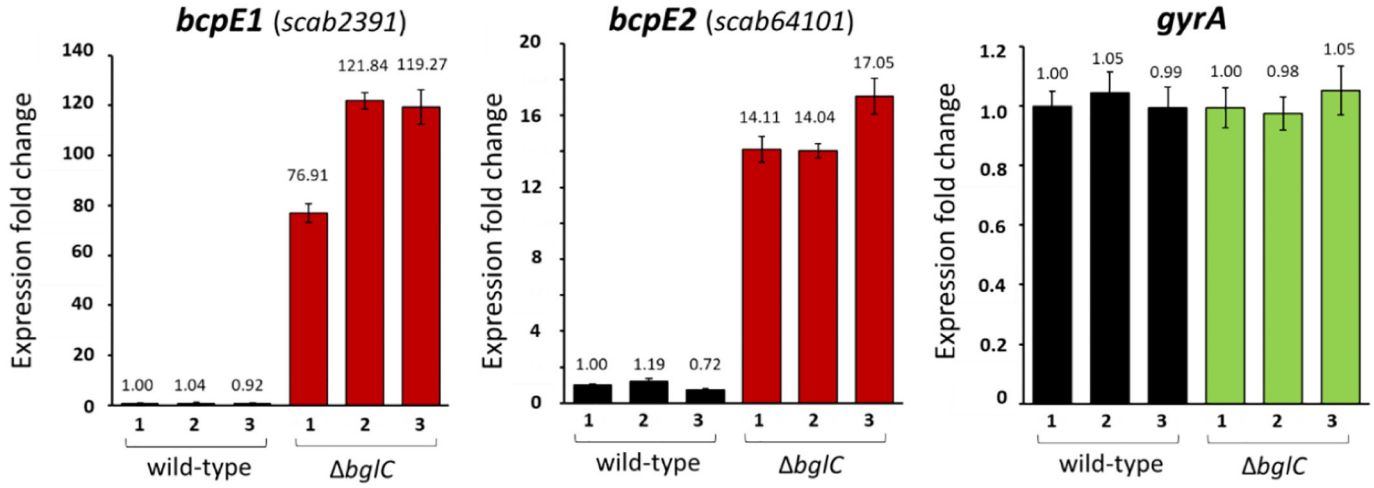


**Fig. 6.** Effect of *bglC* d*eletion on transcription of bcpE1 and bcpE2.* Expression levels of *bcpE1* and *bcpE2* were analyzed and compared in *S. scabies* wild-type and its *bglC* deletion mutant (Δ*bglC*) by quantitative RT-PCR. Relative expression values, normalized to the *gyrA* and *murX* control genes, are means +/− SEM ($n$ = 3) of 3 technical replicates for each cDNA/primer combinations. Values are further displayed as fold change using the first wild-type replicate as reference.

knockout (KO) mutants by PTC introduction is not universal and the molecular machinery responsible for the observed compensation in this case cannot be transposed to prokaryotes. Also, this requires nucleotide sequence homology between the KO gene and the compensatory gene (s) which is not the case for *bcpE* genes and *bglC*. The gene *scab2391* encoding BcpE1 is part of a gene cluster similar to the *cebR-cebEFG-bglC* cluster, *bcpE1* being a paralogue of *bglC*. Due to the divergence of each cluster after the duplication event, *bcpE1* and *bglC* share 75% of nucleotide identity (89% sequence coverage) that corresponds to the lower bound of the threshold value proposed for the involvement of sequence homology-based compensation mechanisms between two paralogues [15]. However, even if such mechanism may exist for *bglC* and *bcpE1*, sequence homology-based interaction is excluded between *bglC*/BglC and *bcpE2*/BcpE2 (between RNA or proteins) suggesting that at least two different compensation mechanisms would coexist in our model.

Whether this genetic compensation also occurs in non-pathogenic streptomycetes and other organisms that possess the orthologue of *bglC* is unknown. The mechanism involved to force the expression silencing of the two BcpE β-glucosidase encoding genes is also unknown. The increased transcription of "*bcpE*" genes is unlikely to imply a mechanism of adaptive mutation as targeted Sanger sequencing of the *scab2391* (*bcpE1*) and *scab64101* (*bcpE2*) genes and their upstream region (including their promoter) in the Δ*bglC* mutant did not reveal any mutation compared to the wild-type sequence. Although we cannot exclude the existence of adaptive mutations elsewhere in the genome, the likelihood of spontaneous mutations occurring systematically in independently generated mutant strains is very small. The PCR targeting system (*Re*-Direct [42]) used to generate the *bglC null* mutants is based on double homologous recombination with large homologous regions and is thus unlikely to induce off-target mutations outside the targeted region.

Fig. 7 illustrates possible means by which either i) the gene *bglC*, ii) the product of its transcription (the *bglC* mRNA), iii) the protein BglC, or iv) the BG activity of BglC could prevent transcription of both *scab2391* and *scab64101*. The involvement of these different possible scenarios – among others – is currently under investigation.

## 4. Conclusion

The work presented here describes the comprehensive methodology that allowed us to i) unambiguously characterize the genetic compensation phenomenon as a result of the deletion of *bglC*, ii) to identify the compensating genes/enzymes, iii) to characterize their enzymatic properties in order to assess their compensating role, and iv) reveal that the GC phenomenon occurred at the transcriptional level. Why a mutant of a gene associated with a simple function - such as the hydrolysis of cellobiose by BglC - does not behave as expected, and instead displays more of the deleted function than the wild-type strain? Why and how does genetic compensation occur? Most phenotypes associated with this phenomenon are still in search of molecular mechanisms. We then propose different possible scenarios that would explain how, in the wild-type strain, the expression of these genes is kept silent. The molecular basis underlying genetic compensation observed in our case study offers the opportunity to uncover the molecular basis of another genetic compensation mechanism, thereby contributing to answer the important questions pending with respect to the consequences following gene loss and how to interpret genetic robustness.

## 5. Materials and methods

### 5.1. Bacterial strains and culture conditions

*E. coli* strains (DH5α or BL21(DE3) Rosetta™ (Novagen)) used in this work were cultured at 37 °C in LB medium (BD Difco LB broth). *Streptomyces scabies* 87–22 (wild-type), and Δ*bglC* (Δ*scab57721*) strains were cultured at 28 °C in the following media: MM (Minimal Medium, for 1 L: 1 g $(NH_4)_2SO_4$, 0.5 g $K_2HPO_4$, 0.2 g $MgSO_4.7H_2O$ and 0.01 g $FeSO_4.7H_2O$, fixed at pH 7.2), TDM (thaxtomin defined medium [30]). Cellobiose was added as 10× (7% *w/v* or 5 mM) concentrated solution to obtain a final concentration of 0.7% w/v or 500 μM depending on the assay. ISP2 (for 1 L: 4 g Yeast Extract, 10 g Malt Extract, 4 g Dextrose, fixed at pH 7.2) or TSB (Sigma-Aldrich, 30 g/L) were used as rich media for pre-culturing of the bacteria to obtain fresh mycelium. When appropriate, media were supplemented with the antibiotics apramycin (50 mg/mL), kanamycin (50 mg/mL), chloramphenicol (25 mg/mL), and/or thiostrepton (25 mg/mL). Cellobiose was purchased from Millipore (or Carbosynth when used in culture), and cellotriose was purchased from Megazyme, while lactose and raffinose were purchased from Sigma-Aldrich.

Growth curves were performed in TDM + Cellobiose 0.7% in 24-deepwell microplates containing 3.3 mL of medium in each well and inoculated with $5*10^6$ spores. Four culture replicates were sampled at each time point and the $OD_{600nm}$ was measured 5 times for each sample with vigorous mixing between each measurement. Samples of 4 mL from two wells were pooled together in a common pre-weighed tube and centrifuged for 10 min at 4000*g*. The pellet was then dried in an oven at 50 °C for 48 h before being weighed to determine the dry biomass.

### 5.2. bglC gene deletion in S. scabies 87–22

Deletion of *bglC* for generating new *bglC* null mutants (mutants #2, #3 and #4) was performed by replacing *orf* scab57721 with the apramycin resistance cassette using the REDIRECT© PCR targeting methodology [42] as previously described [30,33]. The cassette (consisting of an *oriT* and the antibiotic resistance gene *aac*(3)IV for apramycin resistance flanked by FRT sites (FLP-recombinase recognition targets), was generated by PCR using primers imf298 and imf299 with gene-specific homology extensions (Table 1) and pIJ773 (Table 1) as template. See Jourdan et al. 2016[33] and Jourdan et al. 2018[30] for the detailed protocol.

### 5.3. TLC for hydrolysis of cello-oligosaccharides

Substrate specificity was checked by visualizing di- and oligosaccharides hydrolysis on Thin Layer Chromatography (TLC) plates after incubation with the pure histidine-tagged enzymes BcpE1-$His_6$ and BcpE2-$His_6$. Reactions were conducted in 100 μL HEPES buffer (50 mM, pH 7.5) containing 0.45 μM of either enzyme, 9.37 mM of cello-oligosaccharides or raffinose, or 12.5 mM of lactose, and incubated at 30 °C. At each time-point, 15 μL was sampled and the enzyme was inactivated for 5 min at 98 °C. The samples were then spotted on an aluminum-backed Silica plate 60 (Sigma Aldrich). The elution chamber was filled with Chloroform-methanol-acetic acid-water (50:50:15:1 vol/vol). After elution, the plate was dipped in the revelation buffer (sulfuric acid 5% in Ethanol) and was heated on a hot plate until revelation as described by Gao and Wakarchuk [43]. β.

### 5.4. β-Glucosidase activity assays and kinetic enzymatic properties

β-glucosidase activity determined using pNP-glucose as substrate was performed as described previously [30]. The degradation of pNP-glucose was assessed by following the increase of the absorbance at 405 nm caused by the release of para-nitrophenol (pNP). Samples of 95 μL were mixed with 5 μL of p-NPβG (20 mM) in a 96-well plate. The plate was incubated for 10 min at 25 °C followed by the addition of 100 μL of $Na_2CO_3$ (2 M) to stop the reaction. The release of p-NP was measured with a TECAN infinite® 200 PRO (Mannedorf, Switzerland).

Kinetic parameters of pure BcpE1-$_{His6}$ and BcpE2-$_{His6}$ ($K_m$, $k_{cat}$ and $V_{max}$) with cellobiose as substrate were determined by measuring the glucose released at various cellobiose concentrations ranging between

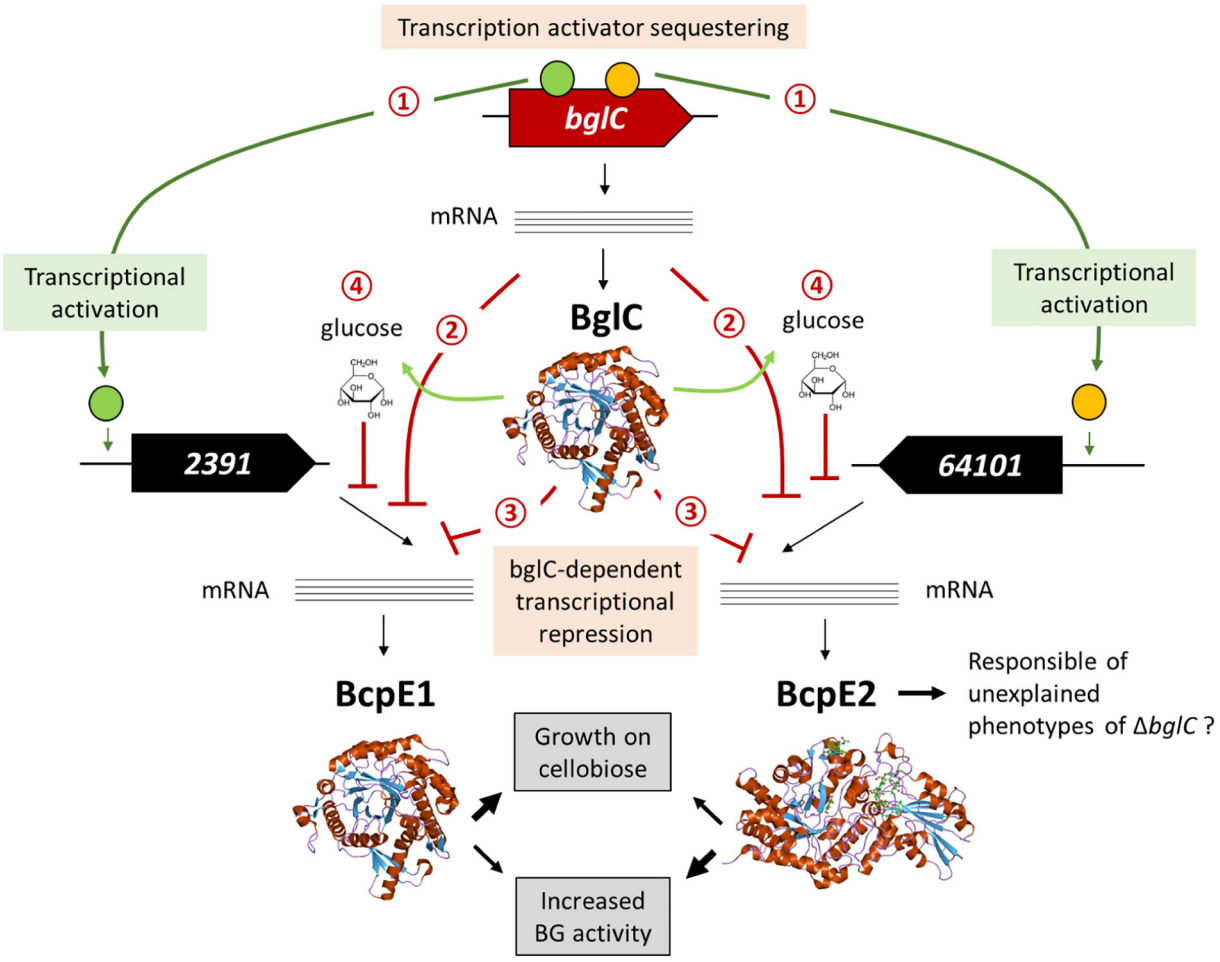


**Fig. 7.** Possible *bglC*/BglC-dependent mechanisms forcing the repression of *bcpE* genes. ① The nucleotide sequence of *bglC* (*scab57721*) would contain DNA-binding sites of transcription activators of *scab2391* (*bcpE1*) and *scab64101* (*bcpE2*). These activators would be sequestered on the *bglC* locus and therefore could not activate transcription of *bcpE1* and *bcpE2*. ② The messenger RNA of *bglC* would prevent the expression of *scab2391* (*bcpE1*) and *scab64101* (*bcpE2*) by RNA-RNA or RNA-protein interactions. ③ The BglC protein would repress transcription of *bcpE* genes by interacting with proteins required for their expression. ④ Glucose generated by the enzymatic activity of BglC or glucose-6-phosphate produced by the glucose kinase GlkA would prevent expression of *bcpE* genes by carbon catabolite repression or another yet unknown process.

0.25 mM and 20 mM in 50 mM HEPES buffer pH 7.5 at 40 °C. Glucose concentrations were measured with the D-Glucose HK Assay Kit (Megazyme) following the manufacturer's guidelines. Absorbance values were measured with a TECAN Infinite® 200 PRO plate reader. Data were fitted to the Henri-Michaelis-Menten equation and linearized (Hanes) using the GraphPad Prism 8 Software.

### 5.5. Fractionation of intracellular β-glucosidases by anion exchange chromatography

A 50-mL ISP2 pre-culture was inoculated with $2 \times 10^7$ spores and incubated 24 h at 28 °C in a shaking incubator (180 rpm). The pre-culture was washed twice in MM. Afterwards, the cells were re-suspended in 50 mL MM + Cellobiose (500 µM) and incubated at 28 °C for 7.5 h. The bacteria were finally centrifuged, and the pellet was re-suspended in 20 mL of HEPES buffer (50 mM pH 7.5) and sonicated 5 times for 30 s with 30-s cooling intervals. The insoluble fraction was removed by centrifugation and the supernatant was filtered (0.22 µm).

The fractionation was conducted on an ÄKTA Prime Plus device with a HiTrap™ Q FF anion exchange chromatography column (GE Healthcare) with a NaCl gradient (0 to 1 M) in order to separate the proteins of the sample in function of their respective pI. The resulting fractions were tested for their β-glucosidase activity against pNP-glucose as substrate and their absorbance at 280 nm was measured in order to assess the total protein amount in each sample. The β-glucosidase activity enrichment was expressed as the $Abs_{405}/Abs_{280}$ ratio.

### 5.6. Heterologous expression and purification of six-histidine-tagged BcpE1 and BcpE2

Genes *scab2391* (*bcpE1*) and *scab64101* (*bcpE2*) were amplified by PCR using specific primers (Table 1) and *S. scabies* 87–22 gDNA as template, and cloned into the pJET1.2 vector generating pBDF001 and pBDF003, respectively (Table 1). *Hind*III and *Nde*I restriction sites were used to clone the BcpE-encoding genes from pBDF001 and pBDF003 to pET22b vectors generating pBDF002 and pBDF004, respectively. Heterologous production of C-terminal six-histidine ($His_6$) tagged BcpE1 and BcpE2 was performed in *E. coli* BL21(DE3) Rosetta™ (Novagen). Cultures for production were carried out in LB medium supplemented with ampicillin (50 µg/mL) and chloramphenicol (15 µg/mL) at 37 °C for 3 h and 18 °C overnight for BcpE1 and BcpE2, respectively.

After resuspension of *E. coli* cells in Tris (50 mM pH 8) buffer supplemented with cOmplete™ EDTA free protease inhibitor cocktail tablets (Roche) and lysis by sonication (5 × 1 min ON/OFF cycles), purification was conducted by affinity chromatography on a 5-mL $Ni^{2+}$- Nitrilotriacetic acid (NiNTA, QIAGEN superflow cartridge) column with an ÄKTA Prime Plus (GE Healthcare) device. Elution of His-tagged BcpE proteins was performed with a linear imidazole

**Table 1**
Primers and genetic constructs used in this study.

| Primers | Sequence (5′ → 3′) | Application |
|---|---|---|
| SCAB_2391_f_ + 1_NdeI | AA*CATATG*CCACCCACCGCACCCGCG | BcpE1-His6 heterologous production |
| SCAB_2391_r_ + 1488_HindIII | AA*AAGCTT*GGCGCCGGGCAGCACGC | |
| SCAB_64101_f_ + 1_NdeI | AA*CATATG*GCGGCAACCCCGGGCACC | BcpE2-His6 heterologous production |
| SCAB_64101_r_ + 2643_HindIII | AA*AAGCTT*CGGTGCCTCGATCGTCACCC | |
| SCAB_2391_fwd_ + 889 | GGAGGACCTGCTCAAGGACA | *bcpE1* qRT-PCR |
| SCAB _2391_rev_ + 968 | GGGTGGGGCTGTAGTAGTTG | *bcpE1* qRT-PCR |
| SCAB _64101_fwd_ + 486 | CACCACCGTCAAGCACTTC | *bcpE2* qRT-PCR |
| SCAB _64101_rev_ + 555 | TCGAAGGGAGCCAGATACAG | *bcpE2* qRT-PCR |
| SCAB _24291_fwd_ + 1306 | GGACATCCAGACGCAGTACA | *gyrA* qRT-PCR |
| SCAB _24291_rev_ + 1366 | CTCGGTGTTGAGCTTCTCCT | *gyrA* qRT-PCR |
| imf298 | CCGTCGGTCGACACGACCACCAATGGGAGCGCTTCCATGATTCCGGGGATCCGTCGACC | *bglC* redirect deletion cassette |
| imf299 | GCTCCCCGGCCCCCGGCTCCGTGGTCCGTCGCTGCCCTATGTAGGCTGGAGCTGCTTC | *bglC* redirect deletion cassette |

| Plasmids/cosmid | Description | Reference/Source |
|---|---|---|
| pJET1.2/blunt | *E. coli* plasmid used for high-efficiency blunt-end cloning of PCR products ($Amp^R$) | Thermo Scientific |
| pET22b | Expression vector used to produce C-terminal $His_6$-tagged protein in *E. coli* ($Amp^R$) | Novagen |
| pBDF001 | *scab2391* (*bcpE1*) cloned in pJET1.2 ($Amp^R$) | This study |
| pBDF002 | pET22b derivative containing the *scab2391* (*bcpE1*) coding sequence from pBDF001 inserted into the *Nde*I and *Hind*III restriction sites ($Amp^R$) | This study |
| pBDF003 | *scab64101* (*bcpE2*) cloned in pJET1.2 ($Amp^R$) | This study |
| pBDF004 | pET22b derivative containing the *scab64101* (*bcpE2*) coding sequence from pBDF003 inserted into the *Nde*I and *Hind*III restriction sites ($Amp^R$) | This study |
| pIJ790 | *E. coli* λ Red plasmid for recombination stimulation ($t^S$, $Cml^R$) | [42] |
| pIJ773 | Template for the REDIRECT© PCR targeting system, contains the [*aac(3)IV* + *oriT*] disruption cassette ($Amp^R$, $Apr^R$) | [42] |
| pUZ8002 | Supplies transfer functions for mobilization of oriT-containing vectors from *E. coli* to *Streptomyces* ($Kan^R$) | [44] |
| Cosmid 833 | SuperCos1 derivative containing the *S. scabies* 87–22 *bglC* gene locus ($Amp^R$, $Kan^R$) | [34] |
| Cosmid 833Δ*bglC* | Cosmid 833 derivative with the bglC gene replaced by the [*aac(3)IV* + *oriT*] disruption cassette ($Amp^R$, $Kan^R$, $Apr^R$) | [30] |

gradient. Content and purity of the collected protein fractions were visualized on 12% SDS-PAGE.

### 5.7. Quantitative RT-PCR

Starting from a 24-h pre-culture in ISP2, mycelium was washed twice with MM before resuspension in MM + Cellobiose (500 μM) and distribution into a 24-deepwell microplate with 2.5 mL of the mycelium suspension per well. After 1 h of incubation at 28 °C shaking at 180 rpm, samples were collected from each well and the NucleoSpin RNA kit (Macherey-Nagel) was used according to the manufacturer's instructions to extract RNA. After verification of the integrity and absence of genomic DNA, the iScript cDNA synthesis kit (Bio-Rad) was used to perform cDNA synthesis. Using a QuantStudio 5 Real-Time PCR system (Thermo Fisher) and a SYBR® Green qPCR MasterMix (Eurogentec), the following 10-μL mix was prepared: 5 μL qPCR MasterMix (2×), 4 μL diluted cDNA (1/10) and 0.5 μL of each specific primer (10 μM). The following protocol was applied to the microplates containing the mixtures: 30 s at 95 °C and 40 cycles of 30 s at 95 °C followed by 45 s at 60 °C, followed by a melting curve analysis heating from 60 °C to 95 °C. Relative expression values, normalized to the *gyrA* and *murX* control genes, are means +/− SEM ($n = 3$) of 3 technical replicates for each cDNA/primer combinations. Data analysis was performed with qBASE+ software (Biogazelle [45])

### 5.8. Targeted proteomics

Fractions collected from the anion exchange chromatography showing the maximum activity in the assay against p-NPβG were analyzed by liquid chromatography – multiple reaction monitoring (LC-MRM) as described previously [30,33,35]. In a preliminary screen, predicted peptides from a panel of potential glycoside hydrolases predicted from the *S. scabies* genome were tested for their presence. Peptides from SCAB_64101, SCAB_2391, SCAB_61811 and SCAB_83501 were found and where then included in the final MRM experiment. Statistical significance ($P < .05$) was assigned by performing two-sided Student's *t*-tests and assuming groups of equal variances.

## CRediT authorship contribution statement

**Benoit Deflandre:** Conceptualization, Methodology, Investigation, Writing – Original Draft, Visualization

**Noémie Thiébaut:** Methodology, Investigation, Visualization

**Sören Planckaert:** Methodology, Formal analysis, Investigation

**Samuel Jourdan:** Conceptualization, Writing – Review & Editing, Supervision

**Sinaeda Anderssen:** Software, Data curation, Writing – Review & Editing

**Marc Hanikenne:** Formal analysis, Resources, Writing – Review & Editing

**Bart Devreese:** Formal analysis, Resources, Writing – Review & Editing

**Isolde Francis:** Resources, Writing – Review & Editing, Supervision

**Sébastien Rigali:** Corresponding author, Conceptualization, Writing – Original Draft, Supervision, Project administration, Funding acquisition

## Declaration of competing interest

The authors declare that they have no conflicts of interest with regard to the publication of this article.

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